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(54) **Monoclonal antibodies and single chain antibody fragments against cell-surface prostate specific membrane antigen**

(57) Isolated monoclonal antibodies or an antigen binding portion thereof which bind to prostate specific membrane antigen in its native form occurring on the surface of tumor cells characterized in that it is linked to

a label or a cytotoxic agent or constructed as a part of a bispecific antibody or a recombinant diabody.

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Description

[0001] Cancer of the prostate is the most commonly diagnosed cancer in men and the second most common cause of death in the Western civilization. No curative treatment currently exists for this tumor after progression beyond resectable boundaries. Because of the significant mortality and morbidity associated with disease progression, there is an urgent need for new targeted treatments. In contrast to cancer in other organ systems, prostate cancer represents an excellent target for antibody therapy for a number of reasons, that include i) the prostate expresses tissue specific antigens, ii) the prostate is a non-essential organ and its destruction will not harm the host, iii) the sites of metastasis are lymph nodes and bone that receive high levels of circulating antibodies, and iv) the PSA serum levels provide a means to monitor therapeutic response.

[0002] Among several candidate markers that have been identified for prostate cancer, prostate specific membrane antigen (PSMA) seems to be most prominent. This type II transmembrane glycoprotein of about 100 KD consists of a short intracellular segment (amino acids 1 - 18), a transmembrane domain (amino acids 19 - 43), and an extensive extracellular domain (amino acids 44 - 750). PSMA may serve as a useful target for immunotherapy because it meets the following criteria: i) expression is primarily restricted to the prostate, ii) PSMA is abundantly expressed as protein at all stages of disease, iii) it is presented at the cell surface but not shed into the circulation, iv) expression is associated with enzymatic or signaling activity. PSMA is also expressed in the neovasculature of most other solid tumors, and therefore may be a target for specific anti-angiogenetic drug delivery.

[0003] Because of their target-oriented specificities, a lot of emphasis has been put on the development of monoclonal antibodies (mAbs) for diagnostic and therapeutic applications in cancer medicine. However, the *in vivo* use of mAbs is associated with serious problems, because of their size and immunogenicity. Therefore, research has focused on the development of smaller therapeutic antibodies with fewer side effects, better tumor accessibility and faster clearance rates. Genetic engineering has made it possible to construct single chain antibody fragments (scFv) which are potentially powerful tools for cancer therapy. These small antibodies are composed of the variable domains of the light chain (V_L) and the heavy chain (V_H) connected by a linker peptide. They show little immunogenicity, almost no toxic effects, an increased clearance rate, an improved uptake by the tumor and a better penetration into the tumor cells. Recombinant murine scFv can be established according to standard methods using either expression libraries from hybridomas or spleen cells of specifically immunized mice [Chowdhury et al., Mol. Immunol. 4 (1997), p. 9-20].

[0004] The first published mAb (7E11-C5) against PSMA targets at the intracellular domain of the protein and was shown to be highly prostate specific [Horoszewicz et al., Anticancer Res. 7 (1987), p. 927-935]. Also, monoclonal antibodies against the extracellular domain of PSMA have been raised after immunization with the antigen. However, there is still a discrepancy between binding to the antigen on fixed cells and histological sections on the one hand and binding to viable tumor cells on the other hand.

[0005] Prostate specific membrane antigen (PSMA) is a prostate marker that is highly expressed in normal prostate as well as in prostate cancer. Its expression is increased in prostate cancer and is found primarily in the prostate.

[0006] Prostate specific membrane antigen (PSMA) is a unique membrane bound cell protein which is over expressed manifold on prostate cancer as well as in the neovasculature of many other solid tumors, but not in the vasculature of the normal tissues. This unique expression of PSMA makes it an important marker as well as a large extracellular target of imaging agents.

[0007] PSMA can serve as target for delivery of therapeutic agents such as cytotoxins or radionuclides. PSMA has two unique enzymatic functions, folate hydrolase and NAALADase and it is found to be recycled like other membrane bound receptors through clathrin coated pits.

[0008] A radio-immuno-conjugate form of the anti-PSMA monoclonal antibody (mAb) 7E11, is commercially available as "ProstaScint[®]" which is currently being used to diagnose prostate cancer metastasis and recurrence. The PSMA epitope recognized by monoclonal antibody 7E11-C5.3 is located in the cytoplasmic domain of the prostate specific membrane antigen.

[0009] There are, however, also reports describing PSMA expression in non-prostatic tissues including kidney, liver and brain. A possible explanation therefore is provided by O'Keefe et al. (Prostate, 2004, February 1; 58 (2) 200-10), namely that there is a PSMA-like gene which possesses 98% identity to the PSMA gene at the nucleotide level, which is expressed in kidney and liver under the control of a different promoter to the PSMA gene.

[0010] WO 01/009192 describes the development of human monoclonal antibodies to prostate-specific membrane antigen. Human anti-PSMA monoclonal antibodies were generated by immunizing mice with purified PSMA. Such purified antigen is a denatured PSMA since it has been purified by immunoadsorption.

[0011] It is one object of the present invention to provide superior means which help to differentiate with higher reliability between tumor cells and healthy cells which do express PSMA or a similar protein but which are not cancer cells. Such constructs can be used to target more specifically tumor cells but not healthy cells.

[0012] Prostate specific membrane antigen (PSMA) is an attractive target for immunotherapy of prostate cancer. However, on prostate cells PSMA is expressed with a specific tertiary and quaternary structure and antibodies elicited

with isolated denatured PSMA do not efficiently recognize PSMA expressing tumor cells. Antibodies and scFv binding to denatured PSMA can be obtained after immunization with the isolated purified antigen. The present invention, however, allows the generation of high affinity antibodies and scFv against native cellular PSMA by a different immunization method which gives only a poor yield of positive clones. Only the later antibodies elicited with native PSMA may react with cell-surface PSMA and can be used as diagnostic and therapeutic tools.

[0013] Monoclonal antibodies (mAbs) and single chain antibody fragments (scFv) of the present invention were prepared according to conventional methods from mice spleen cells. However, the mice had been immunized with LNCaP cells and LNCaP cell lysate containing full-length native PSMA. In a preferred embodiment of the present invention the antigen, namely the full length native PSMA has been obtained after treatment of the cells, preferably LNCaP cells with a special lysis buffer called M-PER, mammalian protein extraction reagent which is commercially available from Pierce, Roquefort, Illinois. The M-PER buffer uses a proprietary detergent in 25 mM bicine buffer (pH 7.6). Hybridomas and scFv were screened and selected by flow cytometry on PSMA-positive LNCaP cells after absorption with PSMA-negative DU 145 prostate cells. Additionally, they were tested for reactivity with purified PSMA. Resulting monoclonal antibodies and scFv were characterized by flow cytometry on LNCaP and PSMA-transfected DU 145 and by western blot with purified glycosylated and deglycosylated PSMA. In addition, immunocytology with LNCaP cells and immunohistochemistry on paraffin sections of prostate cancer samples was prepared.

[0014] In the course of the present invention three mAbs (3/F11, 3/A12 and 3/E7) could be obtained, that were reactive with viable LNCaP cells and PSMA-transfected DU 145 cells but not with other cell lines not expressing PSMA. Binding to LNCaP cells was very strong. At saturation concentrations (100 nM) the mean PE fluorescence intensity (MFI) was between 1000 and 1600. Reactivity with purified PSMA was stronger with the native form (ELISA) than with the denatured and deglycosylated protein (western blot). Immunohistochemistry on paraffin sections was specifically positive for epithelial cells with mAb E7.

[0015] From the mAb 3/A12 two scFv, called E8 and A5, were obtained by selection of recombinant phages on LNCaP cells and purified PSMA. The sequence of scFv E8 was identical to a scFv A4, which was obtained from the B-cell library of the same mouse. ScFv E8 was strongly reactive with LNCaP cells showing a MFI of about 100 at saturation concentrations, whereas the MFI of scFv A5 was only about 40 under the same conditions. No or minimal binding was seen with other cell lines lacking PSMA expression. Binding of both scFv to purified denatured glycosylated and deglycosylated PSMA was weak.

[0016] In the present application we describe three mAb, which are different from those published by other authors with respect to high binding affinity and and high staining of PSMA expressing prostate cancer cells. The antibodies 3/F11, 3/A12 and 3/E7 do not only show a strong binding activity but also internalization into LNCaP cells as shown by immunofluorescence cytology and detection with confocal laser scanning microscopy. These mAbs were obtained after immunisation with full length native PSMA, which is in contrast to different published immunisation methods.

[0017] After immunization with purified denatured PSMA mAbs were obtained which were highly specific for the antigen, but had only a limited binding to PSMA expressing LNCaP cells and also were not internalized into the cells. These control data are not shown in the present application. There are a few anti-PSMA mAbs described in literature. However, the mean fluorescence intensity values were much lower than with the antibodies of the present invention.

[0018] Similarly to the mAbs, anti-PSMA scFv were generated after immunisation with denatured and native PSMA. With the denatured PSMA we obtained scFv highly specific to the antigen, but not binding to LNCaP cells (data not shown in the present application). In contrast, with native PSMA we obtained scFv with a high cell binding activity, but a poor binding to the isolated denatured antigen.

[0019] Because most toxins are intracellularly reactive, only anti-PSMA mAb and scFv that are internalized may be used for generating immunotoxins. There is one publication describing immunotoxins with three anti-PSMA mAbs chemically cross-linked to ricin A-chain [Fracasso et al., Prostate (2002), p. 9-23].

[0020] However, the problems identified in this and other trials with chemically linked immunotoxins are the development of antibodies against the immunotoxins, liver toxicity and vascular leak syndrome and also difficulties in producing large quantities of defined material. These problems are, at least in part, overcome by using recombinant DNA technology which makes the construction of less immunogenic and smaller immunotoxins feasible, and more easily permits the production of immunotoxins in large quantities. It is also believed that penetration into tumors should be better for small proteins than large conjugates. Therefore, two recombinant immunotoxins were engineered by fusing the coding sequence of the scFv E8 and A5 and the toxin PE40. The central finding was that both recombinant immunotoxins effectively killed cultured prostate cancer cells in a dose dependent manner. Strong killing was found not only with the highly binding E8- with IC50 of about 0,05 nM, but also with the lower binding A5-fusion protein with IC50 of about 0,09 nM. Killing of not PSMA expressing prostate cancer cells was 2000-fold less (IC50 = 200 nM). The term IC50 is defined as the concentration in nM of the toxin which reduces cells proliferation to 50% of the cell proliferation without adding a toxin.

[0021] The antibodies and scFv described in this application specifically bind to native cell-surface PSMA and therefore will have value in diagnostic and therapeutic applications focusing on PSMA as a target antigen for prostate cancer.

[0022] Since PSMA is expressed on prostate cancer cells with a specific tertiary and quaternary structure, only anti-

bodies against this cellular conformation may recognize and strongly bind to viable prostate cancer cells and PSMA-expressing tissue. Therefore, the aim of the present study was to generate such mAbs and scFv that can be used for therapeutic and diagnostic targeting of prostate cancer.

[0023] The present invention provides therefore an isolated monoclonal antibody or an antigen binding portion thereof which binds to prostate specific membrane antigen in its native form occurring on the surface of tumor cells which is linked to a label or a cytotoxic agent.

[0024] The term "isolated monoclonal antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains interconnected by disulfid bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, namely CH1, CH2 and CH3. Each light chain contains a light chain variable region (V_L) and a light chain constant region (C_L). The V_H and V_L regions can be further subdivided into regions of hypervariability, which are also called complementarity determining regions (CDR) interspersed with regions that are more conserved. Those regions are also called framework regions (FR). Each V_H and V_L region is composed of three CDRs and four FRs arranged from amino terminus to carboxy terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen.

[0025] In Figures 13 and 14 the CDRs are marked by grey boxes. Those areas are important for the binding of the monoclonal antibody or the antigen binding portion thereof. The other areas are framework regions which can be replaced by other sequences. Monoclonal antibodies derived from mouse may cause unwanted immunological side-effects due to the fact that they contain a protein from another species which may elicit antibodies. In order to overcome this problem the monoclonal antibodies or the antigen binding portions thereof may be humanized. The process of humanizing monoclonal antibodies is known to the person skilled in the art. The framework regions of a mouse mAb are replaced by the corresponding human framework regions. In order to maintain the preferred binding properties the sequences of the CDRs should be maintained as far as possible. It may be required, however, to perform some amino acid changes in order to optimise the binding properties. This can be performed by the person skilled in the art by standard proceedings. Furthermore by introducing a human framework it may be necessary to perform amino acid changes and/or deletions in order to improve the properties of the construct.

[0026] The term "antigen binding portion" of the monoclonal antibody refers to one or more fragments of such an antibody which retained the ability to specifically binding to the prostate specific membrane antigen in its native form. Examples of antigen binding portions of the antibody include a Fab fragment, a monovalent fragment consisting of the V_L , V_H , C_L and C_{H1} domains, an $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfid bridge at the hinge region, an Fd fragment consisting of the V_H and C_{H1} domain, an F_V fragment consisting of the V_L and V_H domains of a single arm of an antibody, a dAb fragment which consists of a V_H domain and an isolated complementarity determining region (CDR).

[0027] The isolated monoclonal antibody or antigen binding portion thereof according to the present invention can preferably be internalized by a tumor cell if it is used for therapeutic purposes. For diagnostic purposes an internalisation may not be required.

[0028] The isolated monoclonal antibody or an antigen binding portion thereof according to the present invention binds strongly to LNCAP cells but not or only minimally to cells which lack expression of prostate specific membrane antigen.

[0029] The binding of the isolated monoclonal antibody or antigen binding portion thereof is measured by PE fluorescence intensity (MFI) which is preferably higher than 40 for an scFv and preferably higher than 1000 for an mAb at saturating concentrations.

[0030] The binding properties of the isolated monoclonal antibodies or an antigen binding portion thereof to the native PSMA were compared by treating LNCAP cells with increasing concentrations of the first step anti-PSMA Ab followed by incubation with the second step PE-labeled antibody. From the resulting saturation curves the antibody concentration reaching 50% saturation of PSMA sites can be read. The three mAb 3/F11, 3/A12 and 3/E7 showed a high binding activity reaching 50% saturation of PSMA sites at approximately 16 nM (3/F11), 2 nM (3/A12) and 30 nM (3/E7). With the scFv a 50% saturation of PSMA sites was found at 10 nM (E8) and 60 nM (A5).

[0031] In order to determine the binding strength the PE (phycoerythrin) fluorescence intensity (MFI) was measured. The MFI values were plotted against the antibody (or binding fragments thereof) concentration whereby the plateau value of MFI corresponds to 100% saturation with antigen. After having determined the top value (plateau corresponding to 100% saturation of antigen) the value corresponding to 50% of saturation can be easily determined. By using the graph the corresponding concentration of the antibodies or binding fragments thereof in nM can be seen.

[0032] The isolated monoclonal antibody or an antigen binding portion thereof comprises a label which may be a particle which emits radioactive radiation. This particle may be a radioactive element in a form which can be linked to the construct, preferably in the form of a complex. For example an mAb labeled with $^{111}\text{Indium}$ may be used as a radioimmunosciintigraphy agent in the detection of distant metastatic tumors in prostate cancer patients.

[0033] Alternatively the isolated monoclonal antibody or antigen binding portion thereof may comprise a cytotoxic agent which is a cell toxic substance selected from the group consisting of toxins, for example taxol, cytochalasin B,

gramicidin D, ethidium bromid, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy antracine dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glyccorticoids, procain, tetracaine, lidokaine, propranolol and/or puromycin.

[0034] In a preferred embodiment of the present invention an isolated monoclonal antibody or an antigen binding portion thereof comprises a partial amino acid sequence of at least 10 consecutive amino acids of SEQ ID NO:1 (scFv E8) or SEQ ID NO:10 (scFv A5). In a preferred embodiment the monoclonal antibody or antigen binding protein thereof comprises at least 25 and more preferred at least 50 consecutive amino acids of SEQ ID NO:1 or SEQ ID NO:10, respectively.

[0035] In a preferred embodiment the isolated monoclonal antibody or antigen binding portion thereof comprises at least one of the CDRs having SEQ ID NO:2 - SEQ ID NO:7 and/or SEQ ID NO:11 to 16. More preferably such construct comprises at least 3 and more preferably at least 5 of those CDR sequences.

[0036] It is a further aspect of the invention to provide DNA sequences which can be used for the preparation of monoclonal antibodies or binding fragments thereof. SEQ ID NO:8 and 9 relate to scFv E8 and SEQ ID NO:17 and 18 relate to scFv A5. The sequences report the coding strand and the complementary strand thereto. SEQ ID NOS:9 and 18 are shown in the 5'→3' orientation. The polynucleotides of the present invention comprise a contiguous sequence of at least 20, preferably 50 and more preferably 100 nucleotides of the group consisting of SEQ ID NOS: 8, 9, 17 and 18. The sequence coding for the CDR are in particular preferred.

[0037] It is one aspect of the present invention to provide a pharmaceutical composition comprising an isolated monoclonal antibody or an antigen binding portion thereof as described in the present application. The pharmaceutical composition of the present invention comprises the monoclonal antibody or an antigen binding portion thereof together with pharmaceutically acceptable additives. Preferably such a composition is prepared for intramuscular or intravenous injection. Alternatively the antibody may be provided in a depot formulation which allows the sustained release of the biologically active agent over a certain period of time which may range preferably from one to six months. Such a sustained release formulation may comprise a biodegradable polymer like a polylactide or polylactide/polyglycolide copolymer which is degraded over a prolonged period of time in the human body whereby the antibody or the antigen binding portion thereof preferably having a toxin is released in a controlled manner over a certain period of time.

[0038] The isolated monoclonal antibody or an antigen binding portion thereof may be used for the preparation of a medicament for the treatment of cancer, in particular prostate cancer.

[0039] Alternatively the invention provides a diagnostic kit for the detection of tumor cells comprising an isolated monoclonal antibody or an antigen binding portion thereof. In such embodiments the label allows the detection of the construct with suitable detection devices.

[0040] The invention provides also a method for the in vitro identification of tumor cells by which the tumor cells to be identified are contacted with an isolated monoclonal antibody or an antigen binding portion thereof which carries a label which can be detected by suitable analytical devices. The label allows the diagnostic identification of tumor cells, for example in section of human tissues obtained after surgery or biopsy.

Brief Description of the Figures

[0041]

Fig. 1: FACS-analysis of the mAb 3/F11, 3/A12 and 3/E7 binding to the surface of PSMA-expressing LNCaP cells at saturation concentrations

Fig. 1a-c: Antigen saturation curves of mAb 3/F11 (a), 3/A12 (b), 3/E7 (c)

Fig. 2: Immunofluorescence cytology: Binding of a) mAb 3/F11 b) mAb 3A/12 c) 3E7 to LNCaP cells. The left pictures show a control staining with 4',6-Diamidino-2-phenylindole (DAPI).

Fig. 3: Immunofluorescence cytology: Internalization of a) mAb 3/F11 b) mAb 3A/12 c) 3E7 in LNCaP cells. The left pictures show control staining with 4',6-Diamidino-2-phenylindole (DAPI).

Fig. 4: Western blot with purified PSMA and the mAbs 3/E7, and 3/A12 and 3/F11

Fig. 5: Western blot with glycosylated and deglycosylated PSMA and mAb 3/A12

Fig. 6: Immunohistochemistry of mAb 3/E7 on a paraffin section of prostate cancer

Fig. 7a/b: FACS-analysis of the scFv E8 (a), and A5 (b) on PSMA-expressing LNCaP cells at saturation concentrations

Fig. 7c/d: Antigen saturation curves of scFv E8 (c) and A5 (d)

Fig. 8: Western blot with purified PSMA and the scFv A5

Fig. 9: immunocytology of scFv E8 on LNCaP cells

Fig. 10: construct of the immunotoxin E8-P40

Fig. 11: Cytotoxic effect of recombinant immunotoxin E8-P40 on LNCaP cells

Fig. 12: Cytotoxic effect of recombinant fusion protein A5-P40 on LNCaP cells

Fig. 13: Sequence of scFv E8. DNA sequence is given as well as amino acid sequence whereby the region of the CDWs is identified by a marked area.

Fig. 14: Sequence of scFv A5.. DNA sequence is given as well as amino acid sequence whereby the region of the CDWs is identified by a marked area.

[0042] The present invention is further illustrated by the following examples.

Example 1

a) Preparation of PSMA

[0043] The human prostate carcinoma cell lines LNCaP, DU 145, PC-3 and HeLa as well as the hybridoma 7E11-C5.3 (IgG1-k, PSMA) were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. LNCaP, DU 145 and HeLa were cultured in RPMI 1640 medium, PC-3 in F12 Nutrimix medium, both supplemented with penicillin (100 000 U/l), streptomycin (100 mg/l) and 10 % FCS at 37 °C in a humidified atmosphere of 5 % CO₂. For the generation of LNCaP cells expressing unglycosylated PSMA on their surface 2 µg/ml tunicamycin (ICN Biomedicals) were added to the medium for 48 h.

[0044] Fixed LNCaP cells were obtained by treatment with 4 % paraformaldehyd for 10 min at RT, and then thoroughly washing with PBS.

[0045] For preparing purified PSMA, 10⁸ LNCaP cells were washed with PBS and then lysed in PBS containing 1 % IGEPAL for 20 min at room temperature. After centrifugation at 10,000 g the supernatant was given on a 7E11-C5 affinity chromatography column (Amersham Biosciences, Uppsala, Sweden) and PSMA was eluted with 100 mM glycine buffer pH 2,5 containing 1 % Triton X-100. After neutralisation the protein was extensively dialyzed with PBS.

[0046] For preparation of deglycosylated PSMA, 1/10 vol glycoprotein-denaturing buffer (BioLabs), was added to the solution with purified PSMA and heated for 10 min at 100 °C. Then 1/10 vol 10 % NP-40 (10 %) and 50 U PNGase per µg PSMA was added and incubated at 37 °C for 1 h.

[0047] For preparation of a LNCaP cell lysate containing full length native PSMA, cells were lysed with M-PER reagent (Pierce) for 10 min and then centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant containing native full length PSMA was collected (M-PER-lysate). The high molecular fraction (100 to 600 KD) of this lysate was separated by HPLC on a Biosil 250 size exclusion column.

b) Transfection of full length PSMA into DU 145 and PC3 cells

[0048] Full length PSMA was cloned in two fragments (fragment 1 from bp 262 to the unique EcoRI restriction site at bp 1573 and fragment 2 from position 1574 to 2512) into the vector pCR3.1 (Invitrogen). Transient transfection was obtained by adding a mixture of 4 µg DNA and 10 µl Lipofectamine (Invitrogen) in 500 µl RPMI medium to 10⁶ cells according to the manufacturer's protocol. After 48 h incubation the transient transfected cells were used for testing.

Example 2

[0049] Immunization of mice

[0050] Four-month old female Balb/c mice were immunized intraperitoneally with 300 µg M-PER lysate from LNCaP cells or with the high molecular HPLC fraction of the lysate, or with 10⁶ LNCaP cells, fixed with 2 % paraformaldehyde. These preparations were mixed 1:1 with complete Freund's adjuvant. Each mouse received 4 or 5 immunizations at 2-week intervals. Four days after the last immunization spleen cells were collected and either used for the preparation of

hybridomas or a B-cell library.

Example 3

Preparation of a B-cell library

[0051] The mouse spleen was washed in phosphate buffered saline (PBS), minced to small pieces, washed again in PBS and then gently homogenized in a "loose-fitting" hand homogenizer. The resulting single cell suspension was overlaid onto Ficoll (Pharmacia, Freiburg, Germany) and centrifuged at 400 g for 20 min at room temperature. Interphase B cells were isolated with CD19 microbeads according to the manufacturer's instructions (Miltenyi, Bergisch Gladbach, Germany). 10^6 B-cells were lysed in 350 μ l of a solution consisting of 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5 % sodium N-lauroylsarcosinate and 100 mM 2-mercaptoethanol.

Example 4

a) Preparation of Hybridomas

[0052] The spleen was aseptically removed and a single cell suspension was prepared in RPMI-1640 medium without serum. The splenocytes were added to SP2/0 myeloma cells at a ratio of 10:1 and the fusion and selection was performed to established procedures [Galfre et al., Nature (1979), p. 131-133].

[0053] Hybridoma supernatants were tested by FACS on LNCaP and DU145 cells and by an ELISA with purified PSMA as solid phase. Monoclonal antibodies were purified using a protein G column (Pharmacia).

b) Isotype determination of the mAbs

[0054] Ig-isotypes of the anti-PSMA mAbs were determined by ELISA using either unlabelled (solid phase) or peroxidase-labeled (tracer) anti-isotype specific antibodies (Southern Biotechnology Associates, Birmingham, AL).

c) Isolation and characterization of anti-PSMA conformational monoclonal antibodies

[0055] From Balb/c mice which were immunized 5 times with the M-PER-lysate from LNCaP cells, spleen cells were fused with SP2/0 cells according to established methods. Positive hybridomas were selected by flow cytometry with LNCaP cells and ELISA on purified PSMA. By this way three positive clones were obtained. The corresponding mAbs with their isotypes were 3/F11 (IgG2a), 3/A12 (IgG1) and 3/E7 (IgG2b).

d) Characterization of mAbs

[0056] By flow cytometry it could be observed that the three mAbs and stained LNCaP cells bind very well with a percentage of positive cells ranging from 95% to 98%. The shape of the curves of fluorescence versus number of events suggested that PSMA is homogeneously distributed within the LNCaP cell population (Fig. 1). To evaluate the binding specificity of the anti-PSMA mAbs, PSMA-negative DU145, PC3 cells, HeLa and Jurkat cells were also stained and analyzed by flow cytometry. All three mAbs did not stain the PSMA-negative cells (percentage of positive cells ranging from 0,04% to 2%).

[0057] The binding properties of the three antibodies were compared by treating LNCaP cells with increasing concentrations of the first step anti-PSMA mAb followed by incubation with a saturating amount of a second step PE-(phycoerythrin)-labeled goat antibody followed by cytofluorometry analysis. At antigen saturation concentrations [100 nM] the corrected mean PE (phycoerythrin) fluorescence intensity was about 1000 for mAb 3A12, and about 1400 for mAb 3F11 and about 1600 for mAb 3E7. As shown for mAb 3A12 the MFI was 5-fold lower on LNCaP cells expressing unglycosylated PSMA (grown with tunicamycin).

[0058] By immunofluorescence cytology and detection with a laser scanning confocal microscope a strong binding of the three mAbs to LNCaP cells (Fig 2) and also an internalization into these cells could be shown (Fig. 3). All mAbs were positive in an ELISA with purified PSMA as solid phase. With denatured PSMA the mAbs showed a signal at about 100 KD in western blot (Fig 4) whereas the blot with deglycosylated PSMA was weak giving a signal at about 84 KD, which corresponds to literature data (Fig. 5).

[0059] Immunohistochemistry on paraffin sections of prostate cancer was positive with mAb 3/E7 but not with mAbs 3/F11 and 3/A12 (Fig. 6). Data are summarized in Table 1.

Table 1: Characterization of 3 monoclonal antibodies against cell-surface PSMA

Hybridoma	Isotype	FACS LNCaP [MFI]*	FACS PSMA-transf.DU* [MFI]	ELISA PSMA	Blot PSMA	Blot degl. PSMA	Immunohistochemistry
3/F11	IgG2a	1400	105	pos	pos	(pos)	neg
3/A12	IgG1	1000	110	pos	pos	(pos)	neg
3/E7	IgG2b	1600	90	pos	pos	(pos)	pos
MFI = mean fluorescence intensity at scFv concentration reaching antigen saturation (background staining with secondary antibody alone is subtracted) (pos) = slightly positive							

[0060] From these data it is concluded that the 3 mAbs show a very strong and highly specific binding to native and denatured PSMA. Although the binding to deglycosylated PSMA is weaker, a sugar specificity can be excluded from the fact that no binding is seen to cells that do not express PSMA.

Example 5

Preparation of a scFv expression library in the phagemid pSEX

[0061] From the B-cell library or from hybridoma cells total RNA and mRNA was isolated with silicagel-based membranes (Rneasy, Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA synthesis was performed at 42°C for 60 min in a final volume of 50 µl which contained 25 µl of denatured RNA, 10 µl 5x buffer (Promega, Heidelberg, Germany), 5 µl of 10 mM dNTP (dATP, dCTP, dGTP, dTTP, Promega), 1,5 µl RNasin (40 U/µl, Promega) 2,5 µl of 150 pM random hexamer primers, and 2,5 µl of AMV reverse transcriptase (10 U/µl, Promega). Then the encoding regions of the heavy-chains and the gamma and kappa chains were amplified by PCR as previously described by Orum et al. [Nucleic Acids Res. (1993), 4491-4498]. For each chain 25 separate reactions were carried out by combining 25 different constant region forward primers with one corresponding reverse primer. The amplified products for the light chains and the heavy chains were purified by agarose gel electrophoresis.

[0062] The PCR products for the light chains were digested with MluI and NotI, and ligated into the phagemid pSEX81 [Dübel et al., Gene (1993), 97-101] using a molar ratio of 1:3 (2 µg vector, 400 ng insert). The products of one ligation were used for the electroporation of 50 µl electrocompetent *E. coli* XL1 blue cells (Stratagene) according to the supplier's protocol. The bacteria were plated on nine 80 mm diameter agarose plates containing 100 µg/ml ampicillin and 0,1 M glucose (SOB-AG) and incubated overnight at 30 °C. Bacteria were isolated by adding 3 ml 2xYT medium on each plate, scraping them off with a sterile glass spreader and pelleted at 3,000 g for 15 min. From these bacteria plasmid DNA was isolated which revealed the VI sublibrary. Then the PCR products for the heavy chain and the VI sublibrary were digested with NcoI and HindIII. Ligation was prepared at a ratio of 3:1 (2 µg sublibrary and 400 ng insert). Transformation by electroporation, plating and collection of transformed bacteria was done as described for the VI sublibrary. From nine 80 mm diameter SOB-AG plates a total of 18 ml V_HV_L library was obtained.

Example 6

Production and selection of antibody-displaying phage

a) Production

[0063] In the V_HV_L library in phagemid pSEX the antibody genes are fused in frame with gene III, which encodes the minor surface protein gIIIp of the filamentous phage. Therefore, production of recombinant phagemid particles displaying the antibody on the surface requires infection of the phagemid-carrying bacterial cell with the replication defective phage M13KO7 [14]. M13KO7 was added to a 10 ml library culture at a multiplicity of 10. After incubation at 37°C for 90 min the cells were pelleted and resuspended in 15 ml 2xYT-medium containing 100 µg/ml ampicillin, 10 µg/ml tetracycline and 50 µg/ml kanamycin. The culture was incubated overnight at 37°C at 250 rpm, then chilled on ice and centrifuged to remove cells. The supernatant containing the phages was mixed with 1/5 volume of an aqueous solution containing 20% PEG 8,000 and 14% NaCl and incubated 1 h at 4°C. Then a centrifugation step of 30 min at 4°C und 6,200 g was

added. The pellet containing the phages was resuspended in 2 ml 10 mM Tris/HCl pH 7,5, 20 mM NaCl, 2 mM EDTA pH 7,5 and used for panning.

b) Panning to select for antigen- and cell-binding clones

[0064] Panning on purified PSMA was done in 96 well Maxi-Sorb microtiter plates (Nunc) which were coated with a solution of purified PSMA (100 μ l/well, 12 μ g/ml PSMA in PBS) and blocked with 4% non-fat milk/PBS. One ml of purified recombinant phages (circa 10^{11}) were incubated in 1 ml 4% non-fat milk/PBS supplemented with 15 μ l 10% Triton X100 for 15 min and then allowed to bind to 8 wells coated with PSMA for 2 h at 37°C. After 20 rounds of washing with PBS/Tween (0,1%) the bound phages were eluted with 0,1 M Glycin-Puffer pH 2,2. For panning on viable LNCaP cells phages were previously absorbed on DU 145 cells. For this procedure 1 ml (circa 10^{11}) recombinant phages were incubated in 2% non-fat milk/PBS for 15 min and then with 10^7 DU 145 cells for 1 h at room temperature on a shaker. Then the cells were centrifuged and the supernatant with non absorbed phages was incubated with 10^6 LNCaP cells for 1 h at room temperature on a shaker. After 10 washing rounds with 2% non-fat milk/PBS and 5 rounds with PBS the bound phages were eluted with 50 mM HCl with subsequent neutralization with 1 M Tris-HCl (pH 7,5). *E. coli* TG1 cells were infected with the eluted phages, plated on SOB-AG plates and incubated overnight at 30°C. An aliquot of the eluate was used for titration. The selection procedure was repeated three to six times.

c) Small scale phage rescue

[0065] From the titration plate 96 individual colonies were isolated and each transferred into one well of a 96-deep-well microtiter plate filled with 500 μ l 2xYT medium containing 100 μ g/ml ampicillin and 0,1 M glucose (YT-AG) and incubated overnight at 37°C (master plate). Then 40 μ l of saturated culture from each well of the master plate were transferred to the corresponding well of a new plate containing 400 μ l of 2x YT-AG medium.

[0066] To each well about 1×10^{10} M13KO7 helper phages were added and incubated on a shaker for 2 hours at 37°C. Then the plate was centrifuged and the pellet suspended in 2xYT medium supplemented with 100 μ g/ml ampicillin, 10 μ g/ml tetracycline, and 50 μ g/ml kanamycin and incubated at 29°C and 240 rpm overnight. After centrifugation the supernatant containing the rescued phagemids was removed and used for phage ELISA and flow cytometry.

d) Phage-ELISA

[0067] Microtiter plates were coated with purified PSMA (1,5 μ g PSMA/ml PBS) overnight and then blocked with 2% non-fat milk/PBS. To each well 200 μ l of rescued phagemids, preincubated 1:1 with 2% non fat-milk/PBS, were added and incubated for 2 h at room temperature. After five washing steps with PBS-Tween, bound phages were detected with 200 μ l/well anti-M13 antibody conjugated to horseradish peroxidase (Pharmacia) for 2 h at room temperature. Development was carried out with 3,3',5',5'-tetramethylbenzidine as substrate.

e) Isolation and characterization of anti-PSMA conformational scFv

[0068] For generation of anti-PSMA conformational scFv a V_HV_L library in the phagemid pSEX was constructed from the B cell library of a mouse immunized with M-PER-lysate of LNCaP cells. This library had a complexity of 10^7 . In a similar way a V_HV_L library was prepared from the monoclonal antibody 3/A12, which was obtained from the same mouse immunized with LNCaP lysate. This V_HV_L library had a complexity of 10^5 . To isolate phages displaying cellular PSMA binding scFv on their surface, six rounds of panning were performed alternatively on LNCaP cells after absorption with DU-145 cells in polystyrene tubes and in microtiter plates coated with 20 μ g/ml purified PSMA. After three, four and six panning rounds isolated phagemid colonies were grown and phage particles were rescued by infection with M13KO7. Analysis of 800 phage clones from the B-cell library by flow cytometry with LNCaP cells and ELISA on purified PSMA showed one positive clone called E8. Out of the V_HV_L library from mAb 3/A12 two positive clones were obtained after the fourth panning round called A4 and A5. By sequencing it was found that A4 was identical to E8.

[0069] The coding region of the scFv E8 and A5 were transferred from the phagemid pSEX into the expression vector pHOG, containing C-terminal c-myc and His-tags. The sequences with the corresponding CDRs are given in Fig. 13 and Fig. 14. The regions coding for the CDR's of the antigen binding portions are marked in Fig. 13 and 14. Those sequences should not be changed whereas the other parts of the sequence which are not marked can be changed. The appropriate three-dimensional structure must, however, be maintained.

[0070] The scFv E8 strongly reacted with viable LNCaP cells as measured by flow cytometry with MFI values of about 100 at saturating concentrations, whereas binding of A5 was much weaker with MFI-values of about 40 at saturating concentrations (Fig 7). In contrast, binding to purified PSMA as solid phase in an ELISA was weak for E8 and somewhat stronger for A5. A similar pattern was seen in western blots with denatured glycosylated and deglycosylated PSMA (Fig.

8). By immunofluorescence cytology with LNCaP cells and detection by confocal laser microscopy a very good binding of the scFv E8 and internalization could be shown (Fig 9). Data of the scFv are summarized in Table 2.

Table 2: Characterization of 2 scFv against cell-surface PSMA

ScFv	Origin	FACS LNCaP [MFI]	FACS PSMA-transf.DU [MFI]	ELISA PSMA	Blot PSMA	Blot degl. PSMA
E8 = A4	B-cell library and mAb A12	100	70	pos	(pos)	(pos)
A5	MAb A12	40		pos	pos	(pos)
MFI = mean fluorescence intensity at scFv concentration reaching antigen saturation (background staining with secondary antibody alone is subtracted) (pos) = slightly positive						

Example 7

ScFv expression and purification

[0071] ScFv fragments were expressed in *E. coli* XL1-Blue (Stratagene) using the secretion vector pHOG 21 which contains the sequences for the His-6 and c-myc-tag in a C-terminal position of the scFv [Kipriganov et al., J.Immunol.Methods (1997), p. 69-77]. *E. coli* bacteria transformed with pHOG plasmids were grown overnight in 2 x YT-AG-medium, then diluted 1:20 and grown as 600 ml cultures at 37°C. When cultures reached OD 0.8, bacteria were pelleted by centrifugation at 1,500 g for 10 min and resuspended in the same volume of fresh YT medium containing 50 :g/ml ampicillin, 0,4 M sucrose and 1 mM IPTG. Then growth was continued at room temperature for 18 to 20 h. Cells were harvested by centrifugation at 5,000 g for 10 min and 4°C. To isolate soluble periplasmic proteins, the pelleted bacteria were resuspended in 5% of the initial volume of ice-cold 50 mM Tris-HCl, 20% sucrose, 1 mM EDTA pH 8.0. After a 1 h incubation on ice, the spheroblasts were centrifuged at 20,000 g at 4°C for 30 min yielding soluble periplasmic extract in the supernatant. The periplasmic extract was concentrated using Amicon YM10 membranes with a 10 kDa cut-off (Amicon, Witten, Germany) followed by thorough dialysis against 50 mM Tris-HCl, 1 M NaCl, pH 7.0.

[0072] Purification was achieved by immobilized metal affinity chromatography. This was performed using a 1 ml column of chelating Sepharose (Pharmacia) charged with Cu²⁺ and equilibrated with a buffer containing 50 mM Tris-HCl and 1 M NaCl, pH 7.0. The periplasmic extract was loaded, washed with twenty column volumes of equilibration buffer containing 30 mM imidazole and then eluted with the same buffer containing 250 mM imidazole. Eluted material was dialyzed against PBS.

[0073] Determination of the protein content was performed with the Micro BCA Protein Reagent Kit (Pierce) according to the manufacturer's instructions.

[0074] Protein induction was obtained with IPTG and the scFv yield from a 600 ml *E. coli* XL1 culture was about 20 µg.

Example 8

Flow cytometry

[0075] LNCaP, DU 145, and PC3 cells were freshly harvested from tissue culture flasks and a single cell suspension was prepared in PBS with 3% FCS and 0,1% NaN₃. Approximately 10⁵ cells were incubated with 50 µl of rescued phagemids, preincubated 1:1 with 2% non-fat milk/PBS, 1 h on ice. After 3 rounds of washing with PBS 25 µl/well anti-c-myc monoclonal antibody 9E10 (10 µg/ml; Becton Dickinson) or when phages were tested 25 µl/well anti-M13 antibody (10 µg/ml; Pharmacia) were added and incubated 40 min on ice. After washing 3 times with PBS the cells were incubated with 100 µl of PE-labeled goat anti-mouse IgG (Becton Dickinson) for 40 min on ice. The cells were then washed again and resuspended in 100 µl of a solution containing 1 µg/ml propidium iodide (Sigma, Deisenhofen) in PBS with 3% FCS and 0, 1 % NaN₃ in order to exclude dead cells. The relative fluorescence of stained cells was measured using a FACScan flow cytometer and the CellQuest software (Becton Dickinson Mountain View, CA).

Example 9*Immunofluorescence cytology*

[0076] LNCaP cells were grown on glass coverslips for 24 hours. For fixation, cells were treated with 2% paraformaldehyde in PBS for 30 min at RT, which does not permeabilize the cell membrane, washed with 1% BSA-PBS, quenched for 10 min in 50 mM NH₄Cl in PBS, and rinsed with 1% BSA-PBS. Primary monoclonal antibody at 4 µg/ml in 1% BSA-PBS was added and incubated for 60 min at 4°C. FITC-labeled goat anti-mouse secondary antibody (2 µg/ml; Southern Biotechnology Associates Inc. Birmingham, USA) was incubated for 30 min and washed extensively with 1% BSA-PBS.

[0077] For internalization experiments the primary antibody was incubated for 30 min at 37°C before fixation of the cells with 2% paraformaldehyde and permeabilization with 0,5 % Triton X100 in PBS.

Example 10*a) Immunohistochemistry*

[0078] Paraffin tissue sections were first deparaffinized and then treated with 0,3% Triton X100 in PBS for antigen retrieval. Kryostat sections were fixed in cold acetone. The sections were treated 30 min at with 3% H₂O₂ and 10 % methanol for quenching of endogenous peroxidase. After blocking with 1% BSA-PBS the primary antibody was added at a concentration of 2 µg/ml and incubated for 1 h at RT. For the scFv a secondary mouse-anti-c-myc antibody was added for 1 h at RT. Then a biotinylated goat-anti-mouse antibody was incubated for 30 min at RT and finally developed with ABC-reagent (Vectastain).

b) Western blot analysis

[0079] Western blot analysis was performed following sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis of purified PSMA and cell lysate from LNCaP cells and transferred to polyvinylidene difluoride membranes. The blots were blocked overnight in PBS containing 5% non-fat milk and incubated with the purified mAbs or scFv at concentrations of 10 µg/ml for 1 h. Then the blots were washed 5 times with PBS-Tween (0,5%) and incubated with horseradish peroxidase conjugated goat anti-mouse IgG for 1 hour at RT. After 5 washes with PBS-Tween (0,5%) the blots were developed by using 3,3',5',5'-tetramethylbenzidine as substrate.

Example 11*Construction, expression and purification of scFv-PE40 proteins*

[0080] The toxin used in our approach was the truncated version of *Pseudomonas* exotoxin (PE40), lacking domain Ia and containing only domains Ib, II, and III [Pastan et al., J.Biol.Chem. (1989), p. 15157-15160]. The DNA with the coding region in the vector pSW200 was obtained from Prof. W. Wels, Frankfurt [Wels et al., Biotechnology (1992), p. 1128-1132]. The DNA fragment from bp position 253 to 613 encoding PE40 was amplified by PCR from plasmid pSW200. The amplified DNA was then ligated into the vector pHOG-His-scFv in a C-terminal position to the scFv using the restriction site XbaI. All cloning steps were performed according to standard methods in *E. coli* XL1 blue and the products were confirmed by sequencing.

[0081] Protein induction of the immunotoxin and purification by IMAC was the same like the scFv. The products were tested and characterized by SDS-page, western blot and flow cytometry.

Example 12*Cytotoxicity of scFv-PE40 Immunotoxins*

[0082] The metabolism of the red tetrazolium salt WST to a water soluble formazan dye was determined according to the manufacturer's instructions (Boehringer). Target cells (LNCaP and DU 145 as control) were seeded at 2.5 x 10⁴/well of a 96-well plate and grown for 24 hours until a confluent cell layer was formed. Various dilutions of the recombinant immunotoxins in aliquots of 50 µl/well were added and the plates were incubated for 48 hours at 37°C, 5% CO₂. After this time the cultures were pulsed with 15 µl/well WST reagent and incubated for 90 min at 37°C, 5% CO₂. Then the spectrophotometrical absorbances of the samples were measured at 450 nm (reference 690 nm). The immunotoxin concentration required to achieve a 50% reduction in cell viability relative to that of untreated control cultures (50%

inhibitory concentration = IC50) was calculated.

[0083] Cytotoxicity assays (WST) with the immunotoxins E8-P40 and A5-P40 were prepared with PSMA expressing LNCaP cells and DU 145 control cells. As shown in Fig. 11 a high cytotoxic effect could be shown with the immunotoxin E8-PE40 on LNCaP cells with a IC50 value of 0.05 nM. In Fig. 12 the cytotoxic effect of the immunotoxin A5-PE40 is shown with an IC50 of about 0.09 nM. The cytotoxic background on not PSMA expressing DU 145 cells was 5% for the E8 construct and only 0.01% for the A5 construct evidencing a very good therapeutic window.

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ccagtagtcc atagccccgt gccgtagtag aggaaaacct cttgtacagt aatacatggc 480
tgtgtcctca gacttcagac tgctcatttg gaggtacagg ttgttcttgg cattgtctct 540
ggagatgggtg aatcgccccct tgataatgtc tgaatagtag gtataataac caccatcact 600
aatgattgcg acccactcca gcctcttttc cggagtctgg cgaacccaat acatataata 660
gtcactgaaa gtgaatccag aggctataca ggagagtttc agggactctc caggcttcac 720

taagcctccc ccagactcca ccaacttcac gtcggccat

759

5

Claims

1. An isolated monoclonal antibody or an antigen binding portion thereof which binds to prostate specific membrane antigen in its native form occurring on the surface of tumor cells **characterized in that** it is linked to a label or a cytotoxic agent.
2. Isolated monoclonal antibody or an antigen binding portion thereof according to claim 1 **characterized in that** it can be internalized by a tumor cell.
3. Isolated monoclonal antibody or an antigen binding portion thereof according to claim 1 or 2 **characterized in that** it binds strongly to LNCAP cells but not or only minimally to cells which lack expression of prostate specific membrane antigen.
4. Isolated monoclonal antibody or antigen binding portion thereof according to claims 1-3 **characterized in that** the PE fluorescence intensity (MFI) is higher than 40 at antigen saturation.
5. Isolated monoclonal antibody or antigen binding portion thereof according to claims 1-3 **characterized in that** the PE fluorescence intensity (MFI) is higher than 1000 at antigen saturation.
6. Isolated monoclonal antibody or an antigen binding portion thereof according to claims 4 and 5 which show a high binding activity to LNCAP cells reaching 50% saturation of PSMA sites at concentrations between 1 nM and 60 nM.
7. Isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1-6 **characterized in that** the label is a particle which emits radioactive or fluorescence radiation.
8. Isolated monoclonal antibody or antigen binding portion thereof according to claims 1-6 **characterized in that** the cytotoxic agent is a cell toxic substance selected from the group consisting of toxins, in particular taxol, cytochalasin B, gramicidin D, ethidium bromid, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy antracine dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glyccorticoids, procain, tetracaine, lidokaine, propranolol and/or puromycin.
9. Isolated monoclonal antibody or an antigen binding portion thereof according to any of the preceding claims **characterized in that** it comprises a partial amino acid sequence of at least 10 consecutive amino acids of SEQ ID NO:1.
10. Isolated monoclonal antibody or antigen binding portion thereof according to claim 9, **characterized in that** it comprises at least one of SEQ ID NO:2 - SEQ ID NO:7.
11. Isolated monoclonal antibody or an antigen binding portion thereof according to any of the preceding claims **characterized in that** it contains a partial amino acid sequence of at least 10 consecutive amino acids of SEQ ID NO:10.
12. Isolated monoclonal antibody or antigen binding portion thereof according to claim 11, **characterized in that** it comprises at least one of SEQ ID NO:11 - SEQ ID NO:16.
13. Pharmaceutical composition comprising an isolated monoclonal antibody or an antigen binding portion thereof according to any of the preceding claims.
14. Use of an isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1-12 for the preparation of a medicament for the treatment of cancer.
15. Diagnostic kit for the detection of tumor cells comprising an isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1-12.
16. A method for the in vitro identification of tumor cells **characterized in that** the tumor cells to be identified are

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contacted with an isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1-12.

17. Use of an isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1 to 12 for the diagnostic identification of tumor cells.

18. Isolated polynucleotide **characterized in that** it comprises a contiguous sequence of at least 20 nucleotides of any sequence of the group consisting of SEQ ID NOS: 8, 9, 17 and 18.

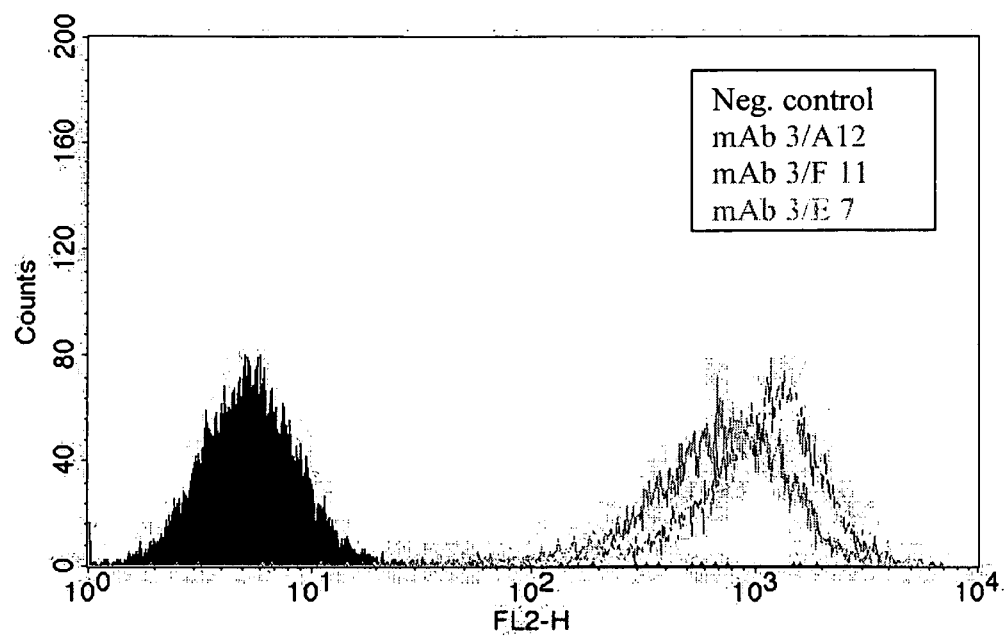


Fig 1

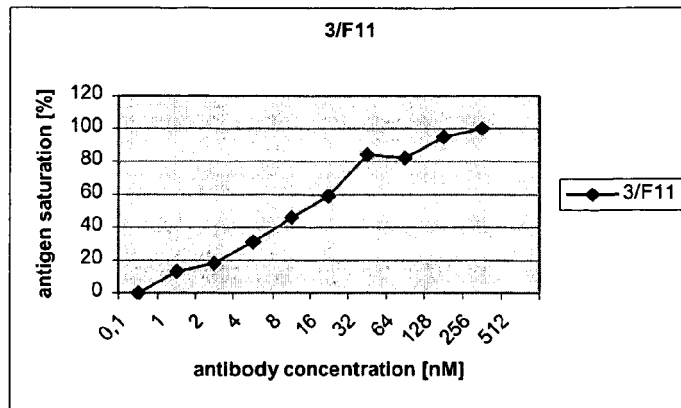


Fig 1a

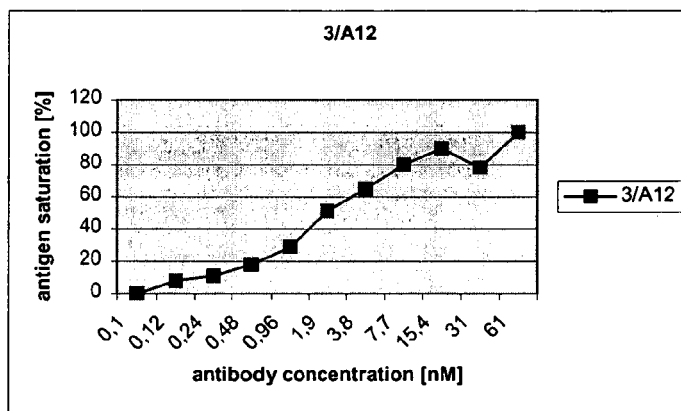


Fig 1b

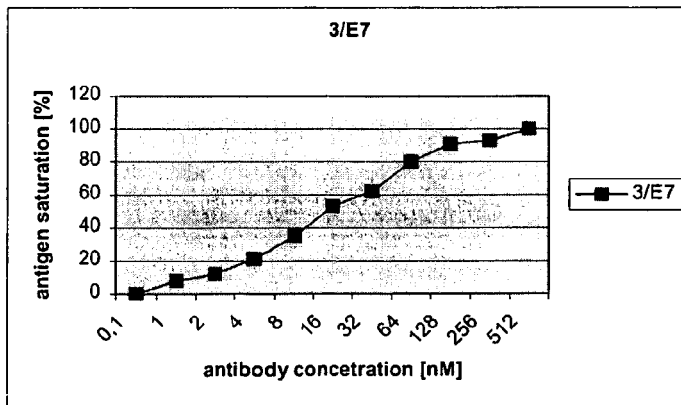


Fig 1c

Fig 1a-c

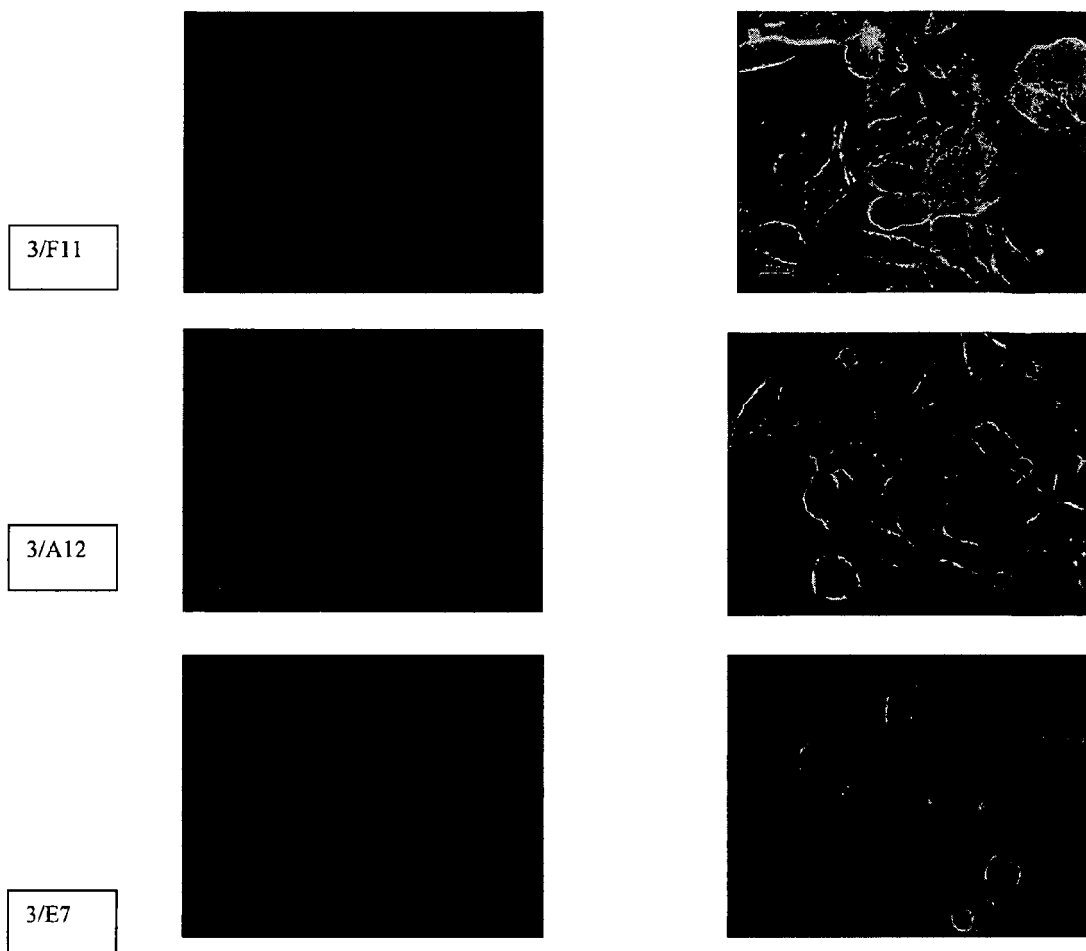
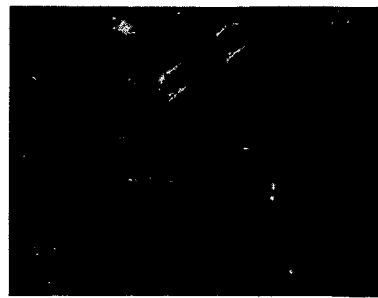
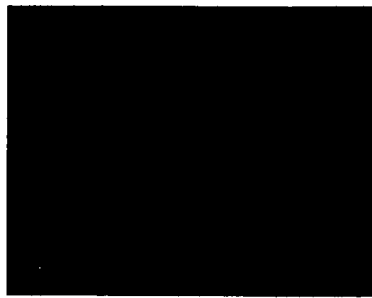
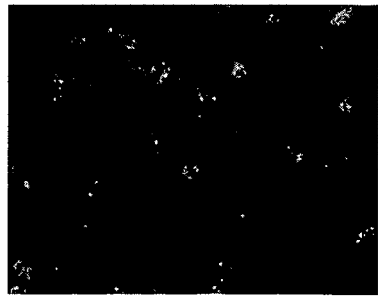


Fig 2

3/F11



3/A12



3/E7

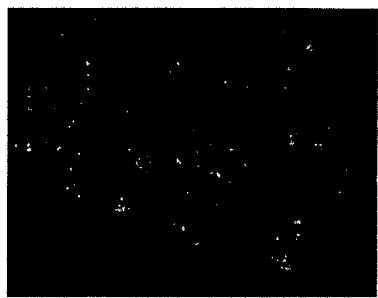
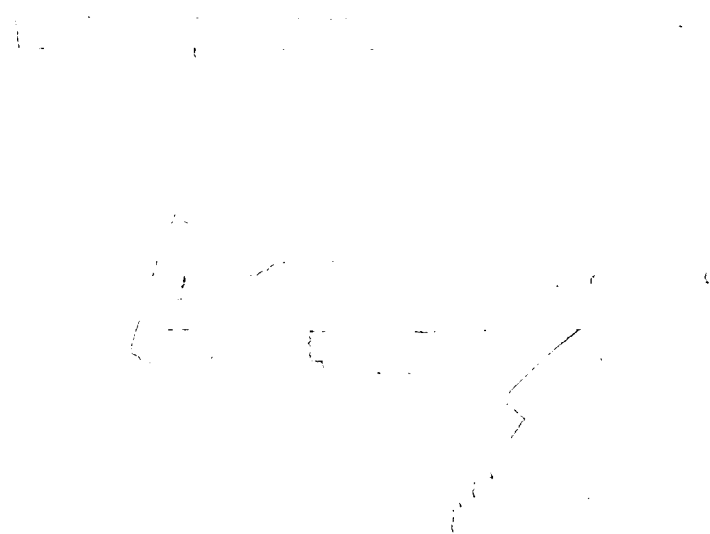


Fig 3



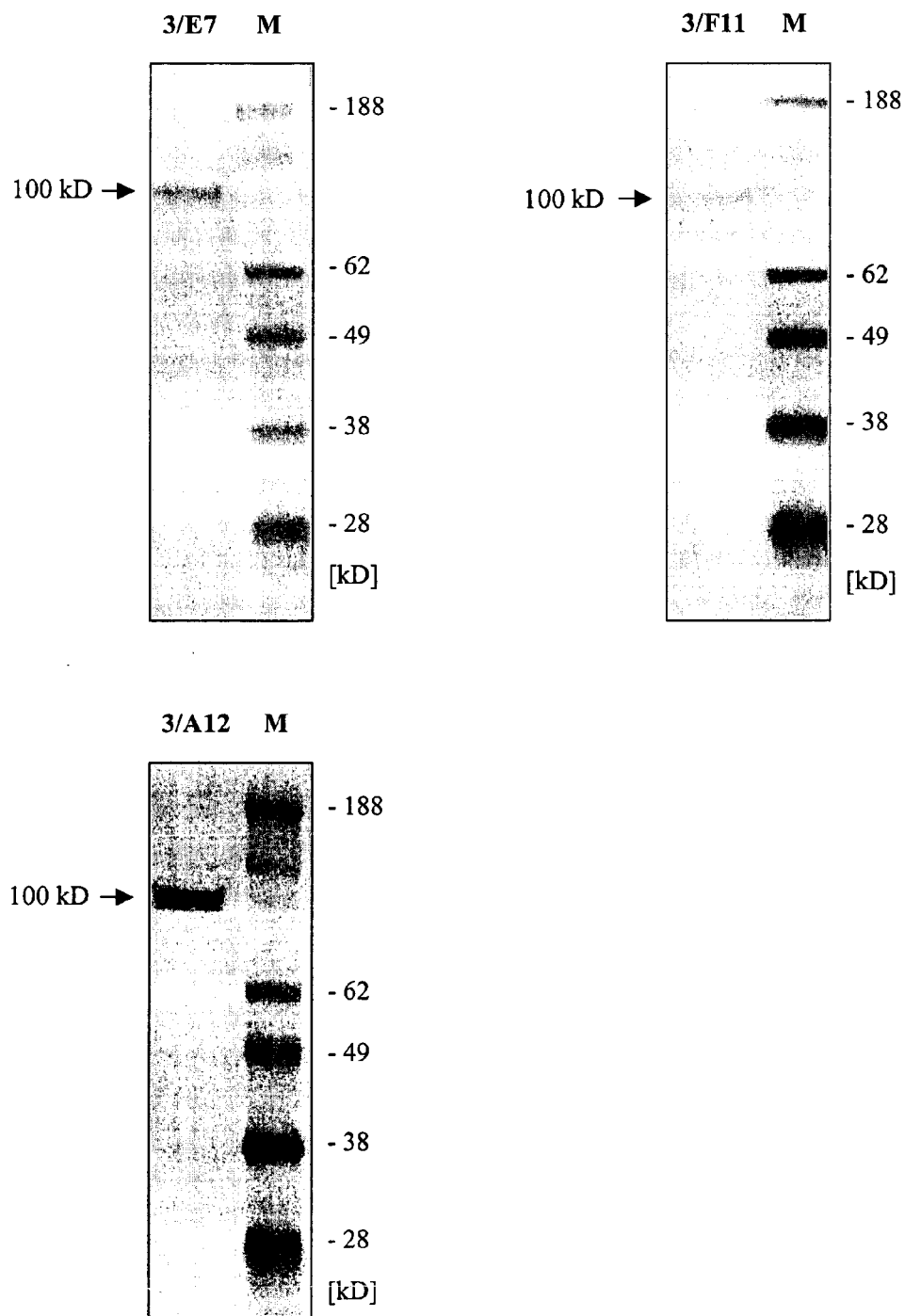


Fig 4

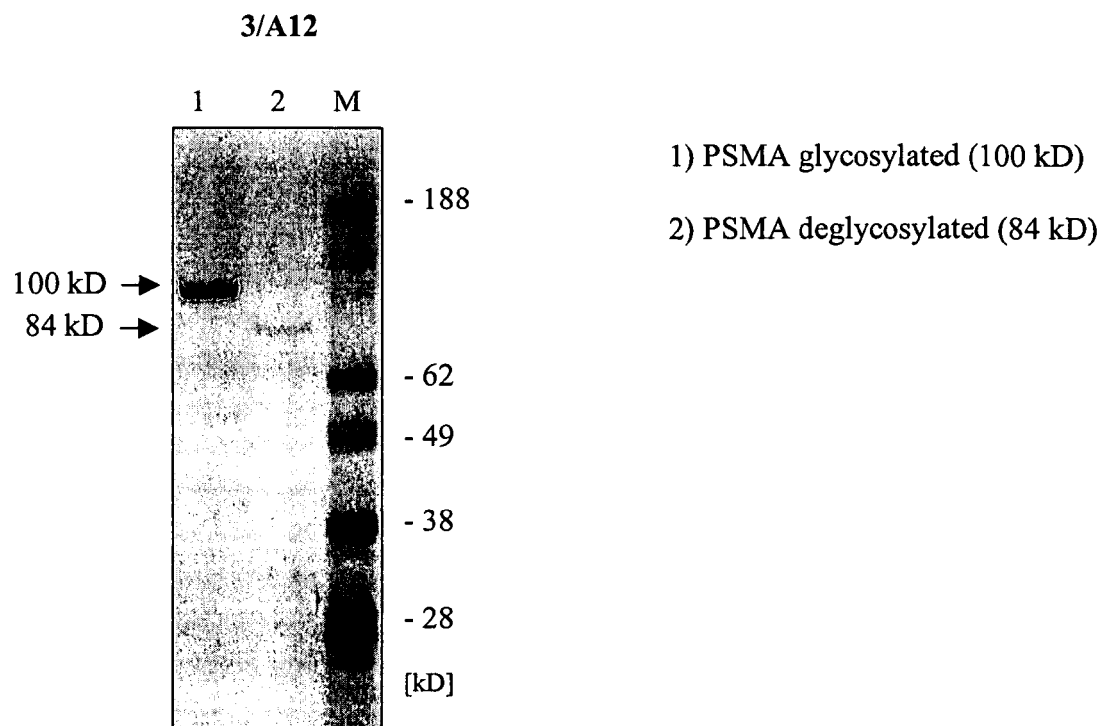


Fig 5



Fig 6

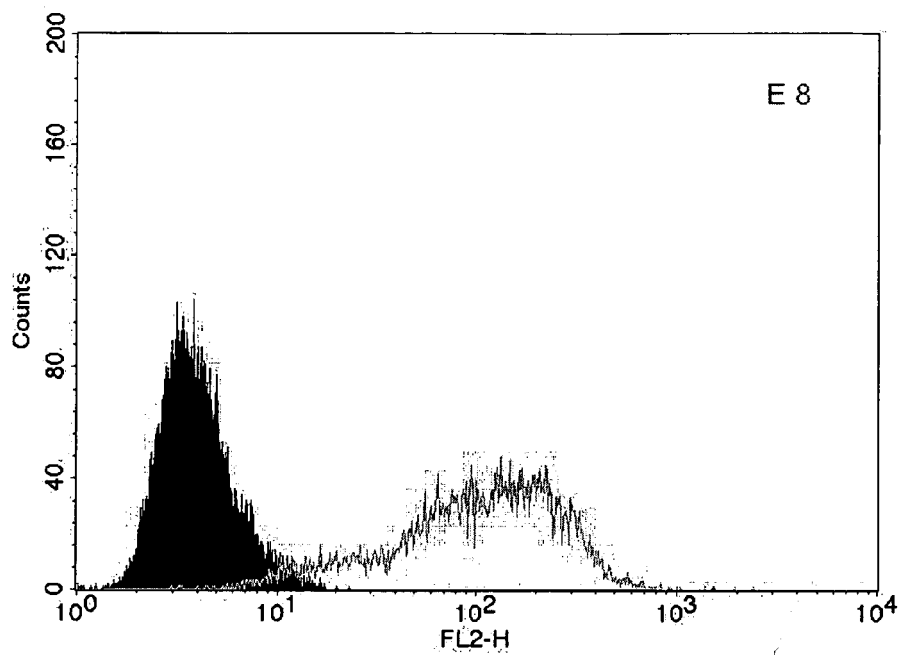


Fig 7 a

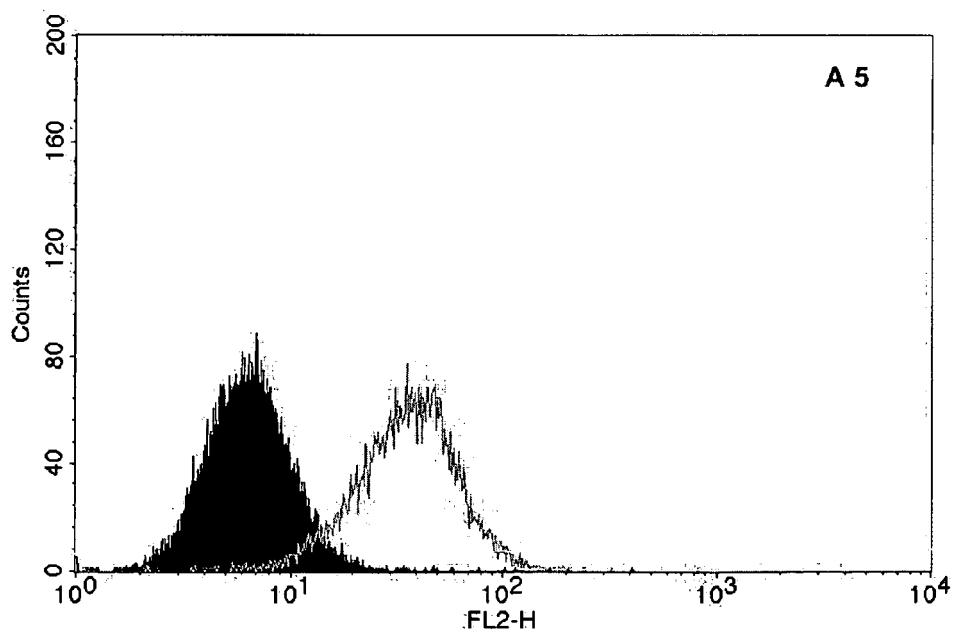


Fig 7 b

Fig 7 a,b

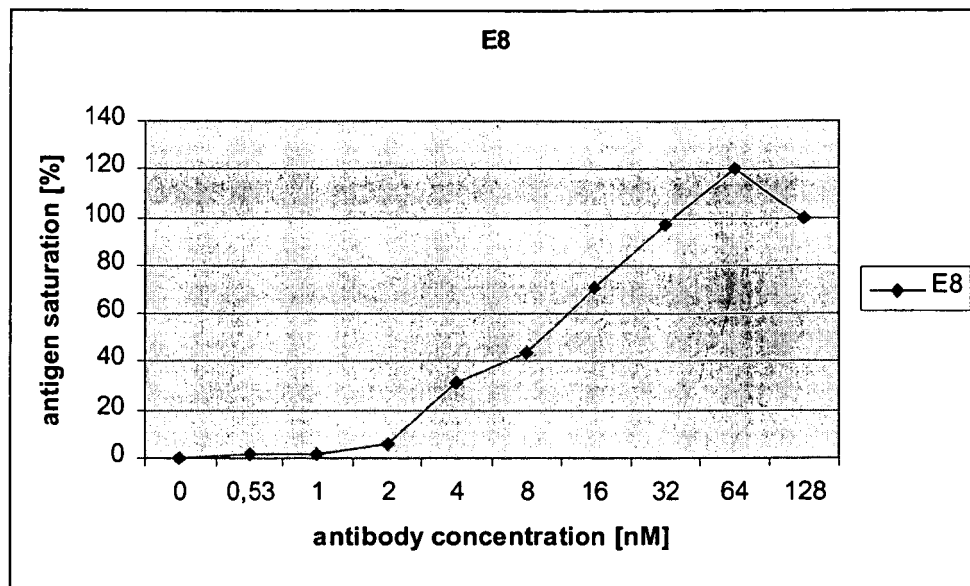


Fig 7c

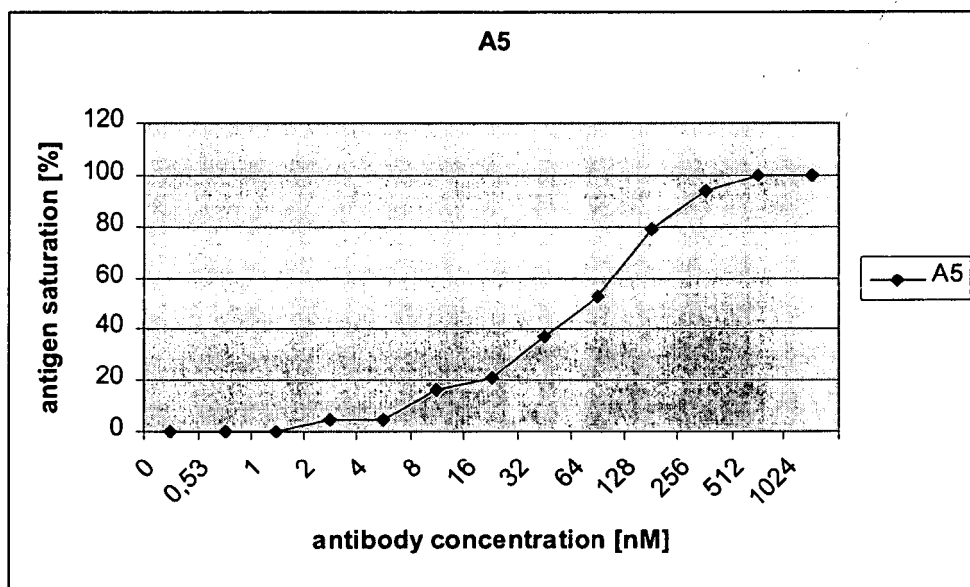


Fig 7d

Fig 7c,d

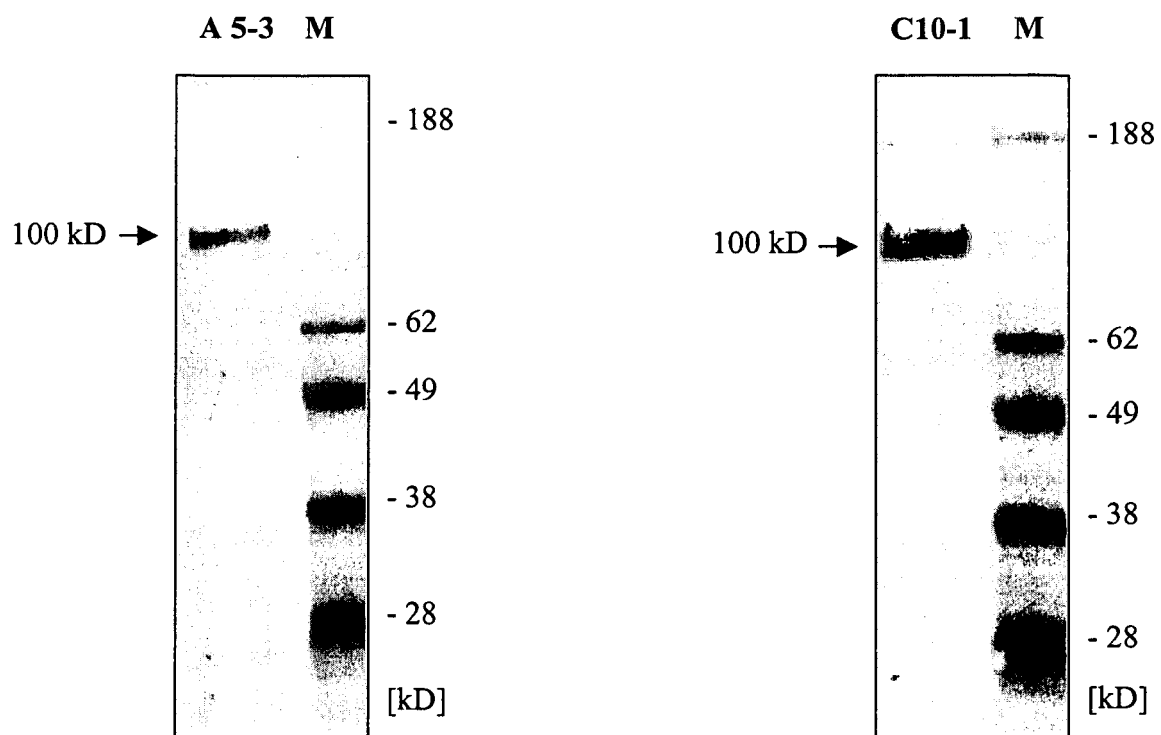


Fig 8

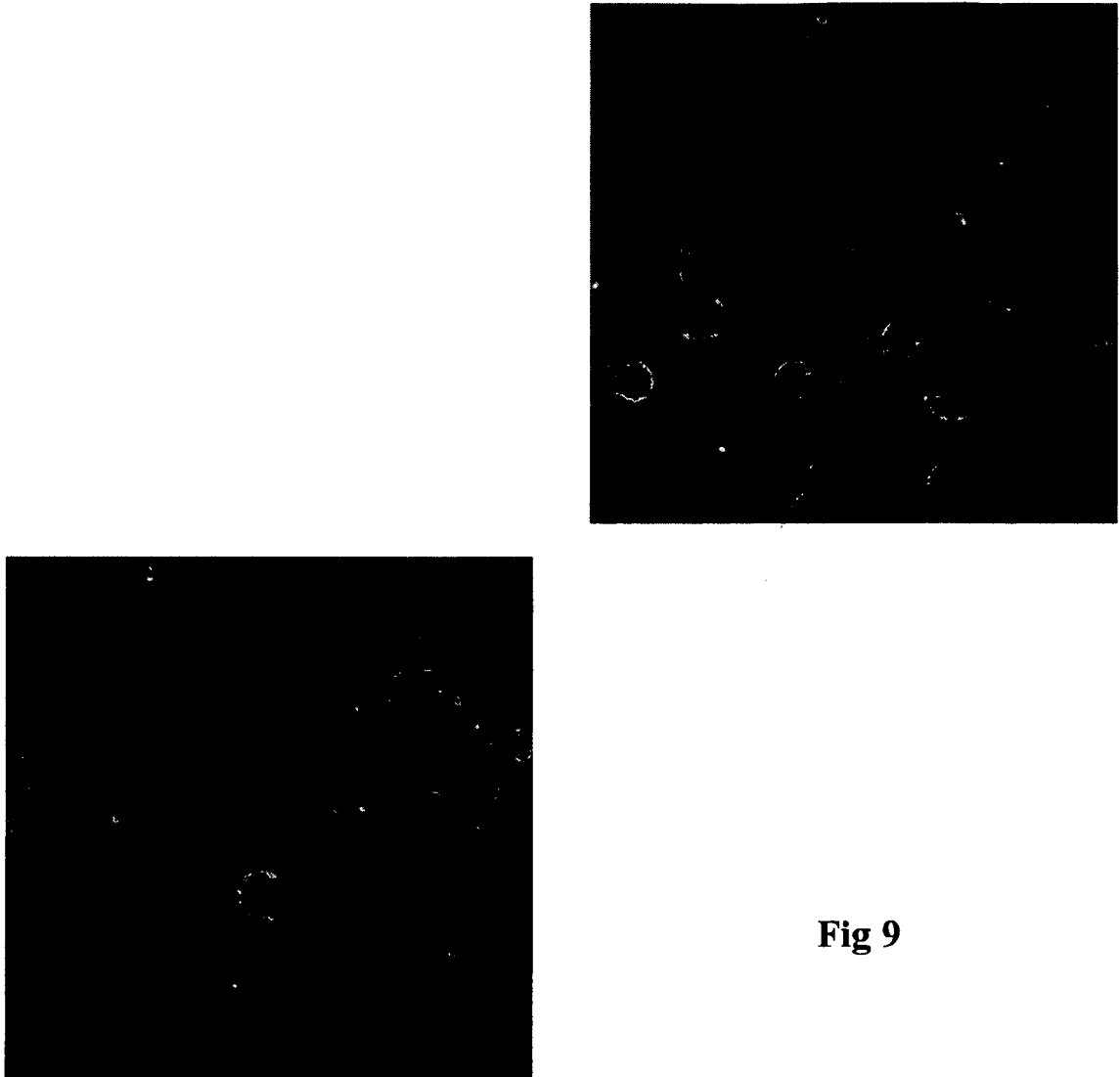


Fig 9

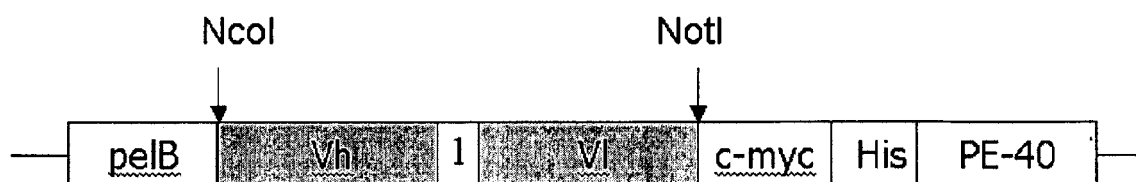


Fig 10

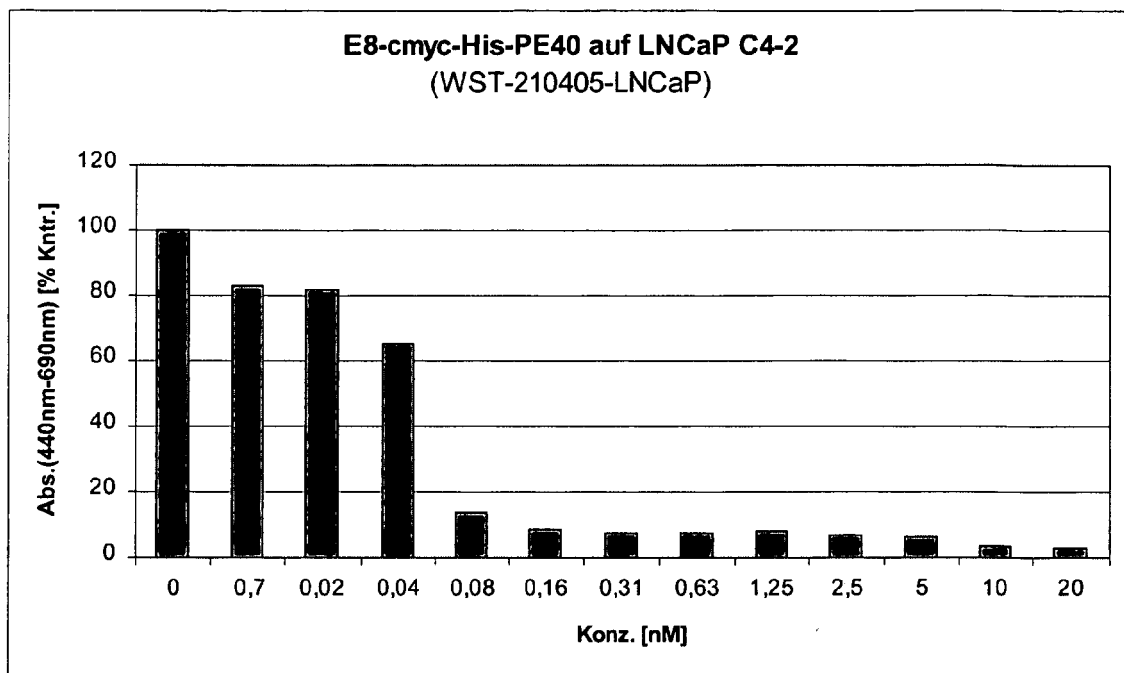
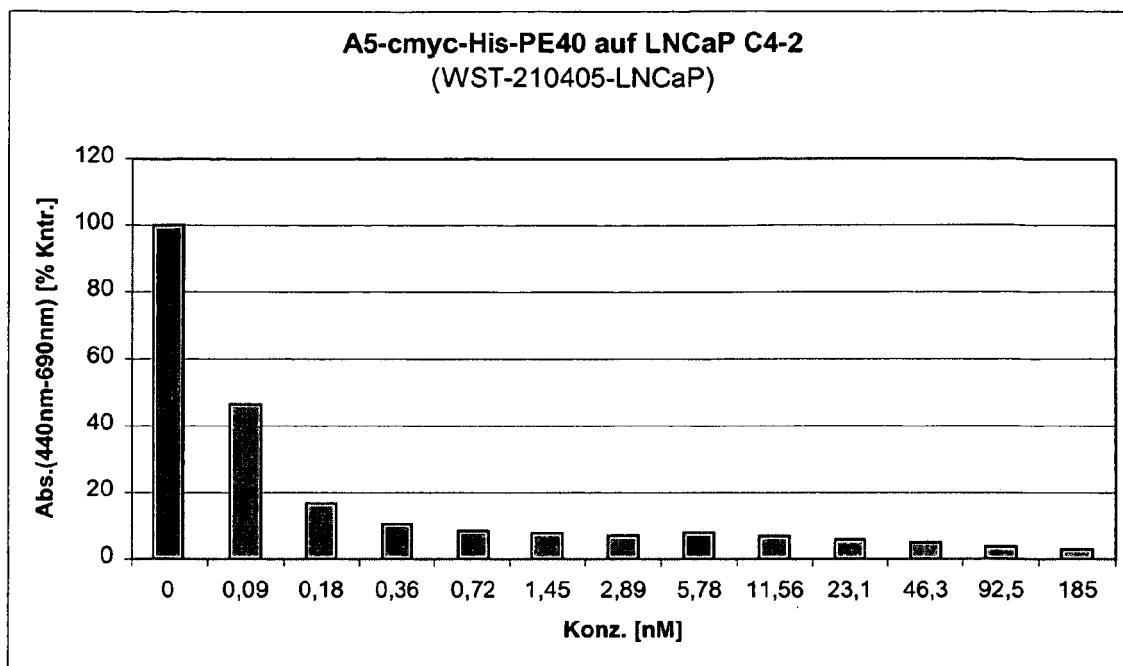


Fig. 11

**Fig. 12**

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      M   A   E   V   Q   L   Q   Q   S   G   P   D   L   V   K   P   G   A
1    ATG GCC GAG GTG CAG CTG CAG CAG TCA GGA CCC GAC CTG GTG AAG CCT GGG GCC
      TAC CGG CTC CAC GTC GAC GTC GTC AGT CCT GGG CTG GAC CAC TTC GGA CCC CGG
      S   M   K   I   S   C   K   A   S   G   Y   T   F   T   D   Y   N   M
55   TCA ATG AAG ATT TCC TGC AAG GCT TCT GGA TAC ACA TTC ACT GAC TAC AAC ATG
      AGT TAC TTC TAA AGG ACG TTC CGA AGA CCT ATG TGT AAG TGA CTG ATG TTG TAC
      D   W   V   K   E   R   H   G   K   S   L   E   W   I   G   D   I   N
109  GAC TGG GTG AAG GAG AGA CAT GGA AAG AGC CTT GAG TGG ATT GGA GAT ATT AAT
      CTG ACC CAC TTC CTC TCT GTA CCT TTC TCG GAA CTC ACC TAA CCT CTA TAA TTA
      P   K   N   G   V   T   I   Y   N   Q   K   F   K   G   K   A   T   L
163  CCT AAA AAT GGC GTT ACT ATT TAC AAC CAG AAG TTC AAG GGC AAG GCC ACA TTG
      GGA TTT TTA CCG CAA TGA TAA ATG TTG GTC TTC AAG TTC CCG TTC CGG TGT AAC
      T   V   D   K   S   S   T   T   A   Y   M   E   L   R   S   L   T   S
217  ACT GTA GAC AAG TCC TCC ACC ACA GCC TAC ATG GAG CTC CGC AGC CTG ACA TCT
      TGA CAT CTG TTC AGG AGG TGG TGT CGG ATG TAC CTC GAG GCG TCG GAC TGT AGA
      E   D   T   A   V   Y   Y   C   A   R   G   D   X   Y   G   N   Y   F
271  GAA GAC ACT GCA GTC TAT TAT TGT GCA AGA GGG GAC TMC TAT GGT AAC TAC TTT
      CTT CTG TGA CGT CAG ATA ATA ACA CGT TCT CCC CTG AKG ATA CCA TTG ATG AAA
      D   Y   W   G   Q   G   T   S   L   T   V   S   S   A   K   T   T   P
325  GAC TAC TGG GGC CAA GGC ACC AGT CTC ACA GTC TCC TCA GCC AAA ACG ACM CCC
      CTG ATG ACC CCG GTT CCG TGG TCA GAG TGT CAG AGG AGT CGG TTT TGC TGK GGG
      YOL epitope 100.0%
      ~~~~~~
      K   L   E   E   G   E   F   S   E   A   R   V   D   I   Q   M   T   Q
379  AAG CTT GAA GAA GGT GAA TTT TCA GAA GCA CGC GTA GAC ATT CAG ATG ACA CAG
      TTC GAA CTT CTT CCA CTT AAA AGT CTT CGT GCG CAT CTG TAA GTC TAC TGT GTC
      S   P   A   S   L   S   V   S   V   G   E   T   V   T   I   T   C   R
433  TCT CCA GCC TCC CTA TCT GTA TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT GGA
      AGA GGT CGG AGG GAT AGA CAT AGA CAC CCT CTT TGA CAG TGG TAG TGT ACA GCT
      T   S   E   N   I   Y   S   N   L   A   W   Y   Q   Q   K   Q   G   K
487  ACA AGT GAG AAT ATT TAC AGT AAT TTA GCA TGG TAT CAG CAG AAA CAG GGA AAA
      TGT TCA CTC TTA TAA ATG TCA TTA AAT CGT ACC ATA GTC GTC TTT GTC CCT TTT
      S   P   Q   L   L   V   Y   T   A   T   N   L   A   D   G   V   P   S
541  TCT CCT CAG CTC CTG GTC TAT ACT GCA ACA AAC TTA GCA GAT GGT GTG CCC TCA
      AGA GGA GTC GAG GAC CAG ATA TGA GGT TGT TTG AAT CCT CTA CCA CAC GGG AGT
      R   F   S   G   S   G   S   G   T   Q   Y   S   L   K   I   N   S   L
595  AGG TTC AGT GGC AGT GGA TCA GGC ACA CAG TAT TCC CTC AAG ATC AAC AGC CTG
      TCC AAG TCA CCG TCA CCT AGT CCG TGT GTC ATA AGG GAG TTC TAG TTG TCG GAC
      Q   S   D   D   S   G   T   Y   Y   C   Q   H   F   W   G   T   P   Y
649  CAG TCT GAT GAT TCT GGG ACT TAT TAC TGT CAA CAT TTT TGG GGT ACT CCG TAC
      GTC AGA CTA CTA AGA CCC TGA ATA ATG ACA GTT GTA AAA ACC CCA TGA GGC ATG
      T   F   G   G   G   T   K   L   E   I   K   R   A   D   A   A   A
703  ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA CGG GCT GAT GCT GCG GCC
      TGC AAG CCT CCC CCC TGG TTC GAC CTT TAT TTT GCC CGA CTA CGA CGC CGG

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Sequence of scFv E8

Fig 13

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      M   A   D   V   K   L   V   E   S   G   G   G   L   V   K   P   G   E
1    ATG GCC GAC GTG AAG TTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA GAG
    TAC CGG CTG CAC TTC AAC CAC CTC AGA CCC CCT CCG AAT CAC TTC GGA CCT CTC
      S   L   K   L   S   C   I   A   S   G   F   T   F   S   D   Y   Y   M
55   TCC CTG AAA CTC TCC TGT ATA GCC TCT GGA TTC ACT TTC AGT GAC TAT TAT ATC
    AGG GAC TTT GAG AGG ACA TAT CGG AGA CCT AAG TCA AAG TCA CTG ATA ATA TAC
      Y   W   V   R   Q   T   P   E   K   R   L   E   W   V   A   I   I   S
109  TAT TGG GTT CGC CAG ACT CCG GAA AAG AGG CTG GAG TGG GTC GCA ATC ATT AGT
    ATA ACC CAA GCG GTC TGA GGC CTT TTC TCC GAC CTC ACC CAG CGT TAG TAA TCA
      D   G   G   Y   Y   T   Y   Y   S   D   I   I   K   G   R   F   T   I
163  GAT GGT GGT TAT TAT ACC TAC TAT TCA GAC ATT ATC AAG GGG CGA TTC ACC ATC
    CTA CCA CCA ATA ATA TGG ATG ATA AGT CTG TAA TAG TTC CCC GCT AAG TGG TAG
      S   R   D   N   A   K   N   N   L   Y   L   Q   M   S   S   L   K   S
217  TCC AGA GAC AAT GCC AAG AAC AAC CTG TAC CTC CAA ATG AGC AGT CTG AAG TCT
    AGG TCT CTG TTA CGG TTC TTG TTG GAC ATG GAG GTT TAC TCG TCA GAC TTC AGA
      E   D   T   A   M   Y   Y   C   T   R   G   F   P   L   L   R   H   G
271  GAG GAC ACA GCC ATG TAT TAC TGT ACA AGA GGT TTT CCT CTA CTA CGG CAC CGG
    CTC CTG TGT CGG TAC ATA ATG ACA TGT TCT CCA AAA GGA GAT GAT GCC CTG CCC
      A   M   D   Y   W   G   L   G   T   S   V   T   V   S   S   T   K   T
325  GCT ATG GAC TAC TGG GGT CTT GGA ACC TCA GTC ACC GTC TCC TCA ACC AAA ACG
    CCA TAC CTG ATC ACC CCA GAA CCT TGG AGT CAG TGG CAG AGG AGT TGG TTT TGC
      YOL epitope 100.0%
      ~~~~~~
      T   P   K   L   E   E   G   E   F   S   E   A   R   V   D   I   Q   M
379  ACA CCC AAG CTT GAA GAA GGT GAA TTT TCA GAA GCA CGC GTA GAC ATT CAG ATG
    TGT GGG TTC GAA CTT CTT CCA CTT AAA AGT CTT CGT GCG CAT CTG TAA GTC TAC
      T   Q   S   P   K   F   M   S   T   S   V   G   D   R   V   S   V   T
433  ACC CAG TCT CCA AAA TTC ATG TCC ACA TCG GTA GGA GAC AGG GTC AGC GTC ACC
    TGG GTC AGA GGT TTT AAG TAC AGG TGT AGC CAT CCT CTG TCC CAG TCG CAG TGG
      C   K   A   S   Q   N   V   D   T   N   V   A   W   Y   Q   Q   K   P
487  TGC AAG GCC AGT CAG AAT CTG GAT ACT AAT GTA GCC TGG TAT CAA CAG AAA CCA
    ACG ATC CCG TCA GTC TTA CAC CTA TGA TTA CAT CCG ACC ATA GTT GTC TTT GGT
      G   Q   S   P   K   A   L   I   Y   S   A   S   Y   R   Y   S   D   V
541  GGA CAA TCT CCT AAA GCA CTG ATT TAC TCG GCA TCC TAC CGG TAC AGT GAC GTC
    CCT GTT AGA GGA TTT CGT GAC TAA ATG AGC CGT AGG ATG GCC ATG TCA CTG CAG
      P   D   R   F   T   G   S   E   S   G   T   D   F   T   L   T   I   S
595  CCT GAT CGC TTC ACA GGC AGT GAA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC
    GGA CTA GCG AAG TGT CCG TCA CTT AGA CCC TGT CTA AAG TGA GAG TGG TAG TCG
      N   V   Q   S   E   D   L   A   E   Y   F   C   Q   Q   Y   D   S   Y
649  AAT GTG CAG TCT GAA GAC TTG GCA GAG TAT TTC TGT CAG CAA TAT GAC AGC TAT
    TTA CAC GTC AGA CTT CTG AAC CGT CTC ATA AAG ACA GTC GTT ATA CTG TCG ATA
      P   Y   T   F   G   G   T   K   L   E   I   K   R   A   D   A   A
703  CCA TAC ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA CGG GCT GAT GCT GCG
    GGT ATG TCG AAG CCT CCC CCC TGG TTC GAC CTT TAT TTT GCC CGA CTA CGA CGC
      A
757  GCC
    CGG

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Sequence of scFv A5

Fig. 14



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 05 01 1536

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The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 21 November 2005	Examiner Renggli, J
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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EPO FORM 1503 03.82 (P04C01)



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EUROPEAN SEARCH REPORT

Application Number
EP 05 01 1536

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The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 21 November 2005	Examiner Renggli, J
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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EPO FORM 1503 03.82 (P04C01)



European Patent
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Application Number
EP 05 01 1536

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The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (IPC)
Place of search		Date of completion of the search	Examiner
Munich		21 November 2005	Renggli, J
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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EPO FORM 1503 03.82 (P04C01)

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 05 01 1536

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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21-11-2005

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